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Potassium-inhibited processing of IL-1β in human monocytes

I.Walev, K.Reske[1], M.Palmer, A.Valeva and S.Bhakdi

Institute of Medical Microbiology and Hygiene, University of Mainz, Hochhaus am Augustusplatz and [1]Institute for Immunology, University of Mainz, Obere Zahlbacher Strasse 67, D-55101 Mainz, Germany

Agents that deplete cells of K^+ without grossly disrupting the plasma membrane were found to stimulate the cleavage of pro-interleukin (IL)-1β to mature IL-1β. Agents examined in this study included staphylococcal alpha-toxin and gramicidin, both of which selectively permeabilize plasma membranes for monovalent ions, the ionophores nigericin and valinomycin, and the Na^+/K^+ ATPase inhibitor ouabain. K^+ depletion by brief hypotonic shock also triggered processing of pro-IL-1β. The central role of K^+ depletion for inducing IL-1β maturation was demonstrated in cells permeabilized with alpha-toxin: processing of pro-IL-1β was totally blocked when cells were suspended in medium that contained high K^+, but could be induced by replacing extracellular K^+ with Na^+, choline$^+$ or sucrose. To test whether K^+ flux might also be important in physiological situations, monocytes were stimulated with lipopolysaccharide (LPS) for 1–2 h to trigger pro-IL-1β synthesis, and transferred to K^+-rich medium. This maneuver totally suppressed IL-1β maturation. Even after 16 h, however, removal of K^+ from the medium resulted in rapid processing and export of IL-1β. Ongoing export of mature IL-1β from cells stimulated with LPS for 2–6 h could also be arrested by transfer to K^+-rich medium. Moreover, a combination of two K^+ channel blockers inhibited processing of IL-1β in LPS-stimulated monocytes. We hypothesize that K^+ movement and local K^+ concentrations directly or indirectly influence the action of interleukin-1β-converting enzyme (ICE) and, possibly, of related intracellular proteases.

Key words: human monocytes/IL-1β maturation/interleukin-1β/interleukin converting enzyme/potassium

Introduction

Interleukin (IL)-1β converting enzyme (ICE) is a recently discovered cysteine protease that cleaves the intracellular 34 kDa IL-1β precursor (pro-IL-1β) to mature 17 kDa IL-1β (Black *et al.*, 1989; Kostura *et al.*, 1989; Cerretti *et al.*, 1992; Thornberry *et al.*, 1992). Generation of IL-1β is coupled to its rapid export via a novel secretory pathway (Rubartelli *et al.*, 1990; Stevenson *et al.*, 1992). ICE is probably a membrane-associated protease (Black *et al.*, 1988) and exhibits DNA sequence homology with the apoptosis-inducing gene *ced3* of *Caenorhabditis elegans* (Miura *et al.*, 1993). The homology includes the region responsible for enzymatic activity in ICE, rendering it probable that the *ced3* gene product is also a protease (Wilson *et al.*, 1994). Indeed, cysteine protease inhibitors can inhibit programmed cell death in T lymphocytes (Sarin *et al.*, 1993). Factors that influence the function of ICE and related proteases are largely unknown. The objective of this communication is to demonstrate that K^+ may represent one such factor.

The experiments reported herein were prompted by our earlier observation that staphylococcal alpha-toxin, a prototype of a bacterial pore-forming cytolysin (Bhakdi and Tranum-Jensen, 1991), appeared to induce rapid processing and secretion of IL-1β from human monocytes (Bhakdi *et al.*, 1989). IL-1β release occurred in permeabilized cells that had been briefly pre-stimulated (60 min) with lipopolysaccharide (LPS), or in cells that had been cultured overnight. We inferred that both treatments led to accumulation of pro-IL-1β in the cells. Toxin-induced IL-1β secretion from these cells was very rapid (complete within 45 min) and did not require *de novo* protein synthesis. At that time, we thought that alpha-toxin created pores of 1–1.5 nm diameter in the plasma membrane, and speculated that influx of Ca^{2+} through the lesions might activate the maturation process (Bhakdi *et al.*, 1989).

Recently, however, it was found that pores created by alpha-toxin in membranes of nucleated cells were smaller than originally thought (Walev *et al.*, 1993). When applied at the low doses at which IL-1β secretion had been observed, alpha-toxin generated pores that permitted only the flux of monovalent ions, and not of Ca^{2+} in lymphocytes (Jonas *et al.*, 1994) and keratinocytes (Walev *et al.*, 1993). Analogous experiments confirmed this finding for human monocytes (our unpublished data), rendering the hypothesis that Ca^{2+} influx triggered IL-1β maturation untenable.

Consequently, experiments were conducted to test whether flux of the monovalent ions might be important. Three main approaches were employed. First, the ionic requirements for processing of pro-IL-1β in cells permeabilized with alpha-toxin were studied. Second, diverse agents that depleted cells of K^+ were tested for their ability to trigger IL-1β maturation. Third, the possible role of K^+ movement was tested in a physiological setting of LPS-stimulated monocytes. The collective data all speak for an important role of K^+ ions in controlling the maturation process. While this manuscript was in preparation, one other report appeared showing that K^+-depleting agents triggered IL-1β maturation (Perregaux and Gabel, 1994). Our results extend those data and provide a working hypothesis that K^+ movement also controls ICE activity in intact cells.

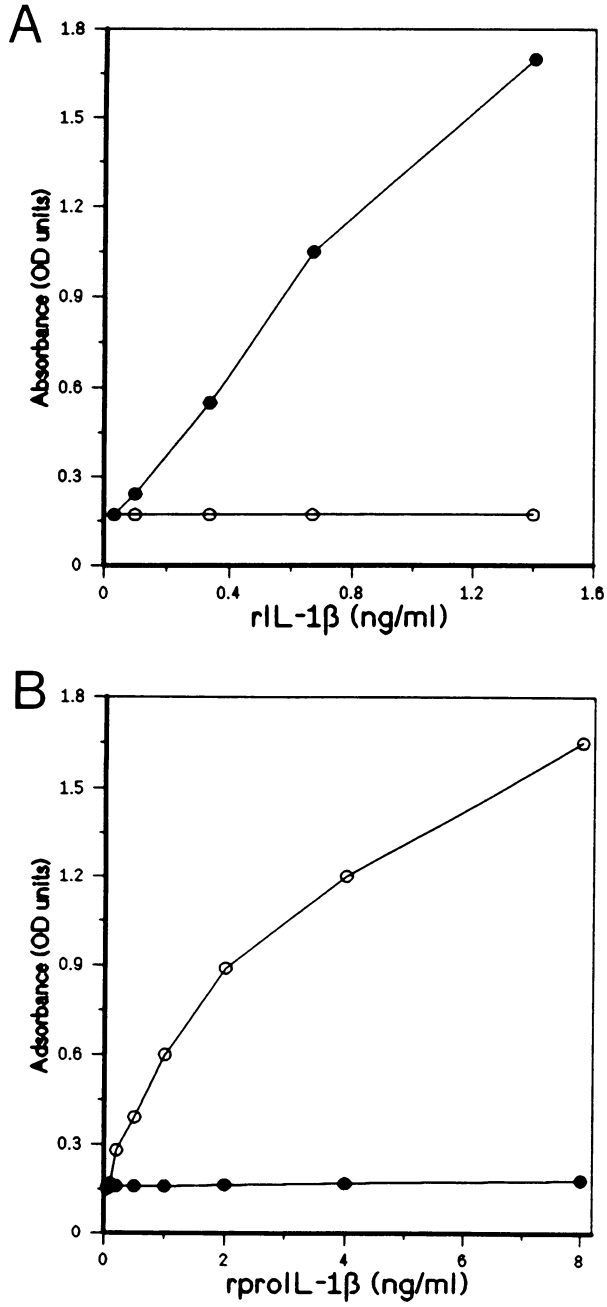

Fig. 1. Specificity of the enzyme immunoassays. (**A**) Measurements performed with the IL-1β ELISA (●) or pro-IL-1β ELISA kit (○). Mature rIL-1β employed as antigen. (**B**) Measurements performed with the IL-1β ELISA (●) or pro-IL-1β ELISA kit (○). r-pro-IL-1β was used as antigen. Note that each ELISA recognized a homologous antigen only.

Results

Specificity of immunoassays employed

The specificities of the two enzyme-linked immunoassay (ELISA) kits were determined by the use of the recombinant proteins supplied with each kit. Dose–response calibration curves are shown in Figure 1, and it is apparent that each ELISA detected only the homologous antigen, i.e. the pro-IL-1β ELISA recognized pro-IL-1β, but not mature IL-1β, and vice versa. These results are in accord with the published data on the IL-1β assay (Bailly *et al.*, 1994), and with unpublished data on the specificity of the pro-IL-1β ELISA (manufacturer).

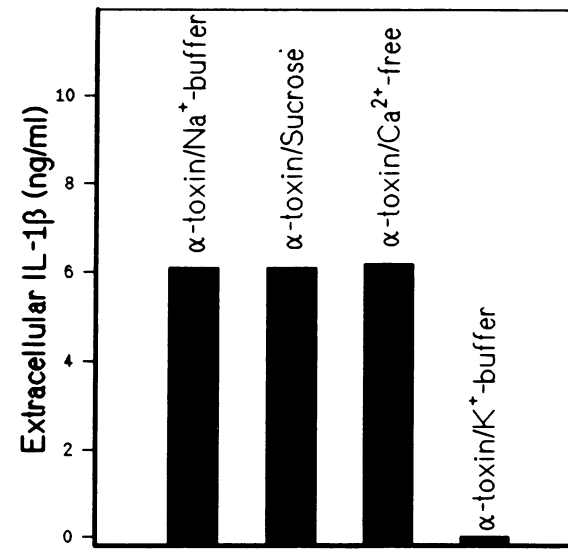

Fig. 2. Extracellular K^+ totally inhibits IL-1β maturation in alpha-toxin-permeabilized monocytes. Cells were stimulated with LPS for 90 min and then transferred either to Na^+ buffer, 20 mM HEPES/260 mM sucrose, Na^+ buffer without Ca^{2+} with 2 mM EDTA or K^+ buffer and permeabilized with 1 μg/ml alpha-toxin. After 60 min, the supernatants were assayed for extracellular IL-1β. Neither Na^+ nor Ca^{2+} influx were required for triggering IL-1β maturation. No maturation occurred in the presence of KCl in the medium.

Ionic requirements for release of IL-1β from cells permeabilized with alpha-toxin

Monocytes were stimulated with 1 μg/ml LPS for 90 min at 37°C. As shown below, this led to the synthesis and intracellular accumulation of pro-IL-1β. It is known that processing and secretion follow in a temporally distinct phase, requiring another 1–2 h (Giri *et al.*, 1985; Burchett *et al.*, 1988; Rubartelli *et al.*, 1990). Therefore, mature IL-1β was not yet present in cell supernatants after 90 min of stimulation with LPS.

The stimulated monocytes were transferred to fresh medium of varying ionic composition. Cycloheximide (CH) was added to stop protein synthesis. Alpha-toxin was applied for 45 min and the supernatants then assayed for IL-1β. As shown in Figure 2, high levels were detected in supernatants of cells treated with alpha-toxin in Na^+ buffer, in 20 mM HEPES/260 mM sucrose, and in Na^+ buffer without $CaCl_2$ supplemented with 2 mM EDTA. Alpha-toxin in choline chloride buffer also triggered IL-1β export (not shown). In contrast, IL-1β was not secreted from cells in the presence of K^+ buffer. Determinations of cellular K^+ showed that cells treated with toxin in the presence of Na^+ buffer and sucrose buffer had residual K^+ contents of 5 and 9%, respectively, compared with controls without toxin after 60 min. These results indicated that neither Na^+ nor Ca^{2+} influx was required for IL-1β maturation and export to occur. At the same time, the results provided the first indication that hindrance of K^+ exit from the permeabilized cells by high extracellular K^+ might be the decisive factor that inhibited the processing step.

This contention was borne out in the next experiments. LPS-stimulated cells were incubated in Na^+ buffer or K^+ buffer with or without 1 μg/ml alpha-toxin. After 90 min, immunoassays were performed on cells and cell supernatants. Control cells harbored large amounts of

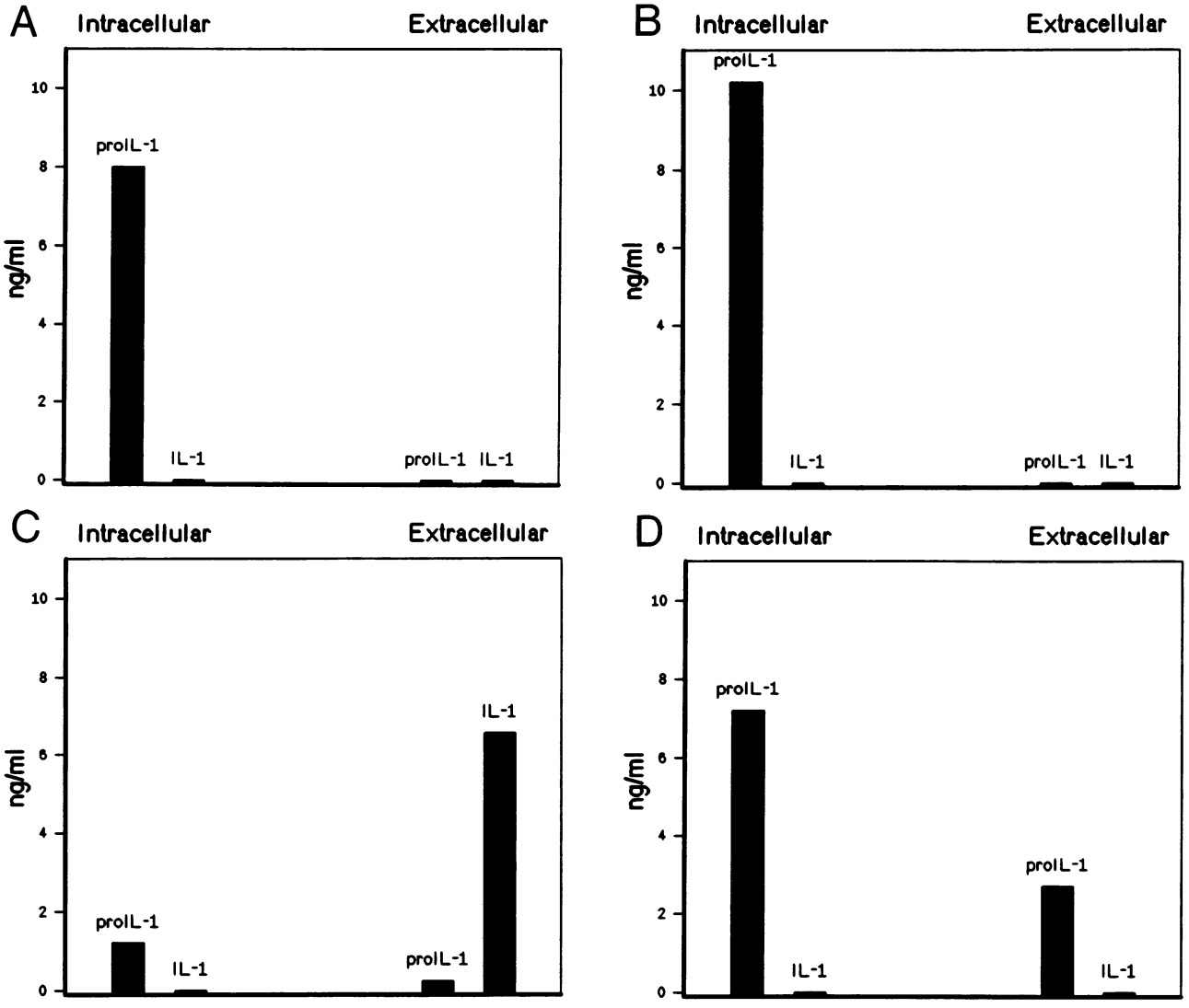

Fig. 3. Measurements of extracellular and intracellular IL-1β and pro-IL-1β. Cells were stimulated for 90 min with LPS, washed, and incubated for another 60 min in Na^+ buffer or K^+ buffer with or without alpha-toxin. Supernatants and detergent-lysed cells were assayed for pro-IL-1β and IL-1β. (**A**) LPS pre-stimulated cells in Na^+ buffer without alpha-toxin. (**B**) LPS pre-stimulated cells in K^+ buffer without alpha-toxin. (**C**) As (A), + alpha-toxin. Note IL-1β processing and secretion. (**D**) As (B), + alpha-toxin. Note the absence of processing and leakage of the IL-1β precursor to the extracellular medium.

pro-IL-1β and only low levels of mature IL-1β were detected in the supernatants (Figure 3A and B). Treatment with alpha-toxin in Na^+ buffer was followed by a rapid and essentially quantitative processing and export of IL-1β, which was recovered solely in supernatants in the mature form (Figure 3C). In contrast, cells incubated with alpha-toxin in the presence of K^+ buffer entirely failed to secrete mature IL-1β. Instead, partial leakage of pro-IL-1β to the supernatants was detected (Figure 3D).

Two-dimensional gel electrophoreses were performed to confirm the validity of the immunoassays. Monocytes were stimulated with LPS for 90 min and then incubated with [^{35}S]methionine for 90 min. The overall longer stimulation period was employed in order to optimize incorporation of the label. Unstimulated cells served as controls. The cells were then transferred to either Na^+ buffer or K^+ buffer, and treated with 1 μg/ml alpha-toxin. A control was left in Na^+ buffer without alpha-toxin. After 40 min, the supernatants were subjected to two-dimensional PAGE. The autoradiographs are shown in Figure 4A, C and E. Following a 21 day exposure, the autoradiographs revealed a large number of spots. However, one prominent 34 kDa entity was present in the supernatants of cells that had been incubated with alpha-toxin in K^+ buffer (Figure 4A) which was absent in the Na^+ buffer supernatant (Figure 4C). On the other hand, the very prominent 17 kDa spot present in the Na^+ buffer/alpha-toxin supernatant (Figure 4C) was almost absent in the K^+ buffer/alpha-toxin supernatant (Figure 4A). The same 17 kDa entity was present in smaller amounts in supernatants of cells that has been incubated in Na^+ buffer without alpha-toxin (Figure 4E). Cells that had not been stimulated with LPS secreted only very low amounts of the 17 kDa polypeptide and none of the 34 kDa protein in the presence or absence of alpha-toxin (not shown).

The 34 and 17 kDa polypeptides were identified as pro-IL-1β and mature IL-1β, respectively, by analyses employing co-electrophoresis of radioiodinated markers. First, a mixture of the two markers was analyzed alone. Pro-IL-1β generated a 34 kDa spot, whereas mature,

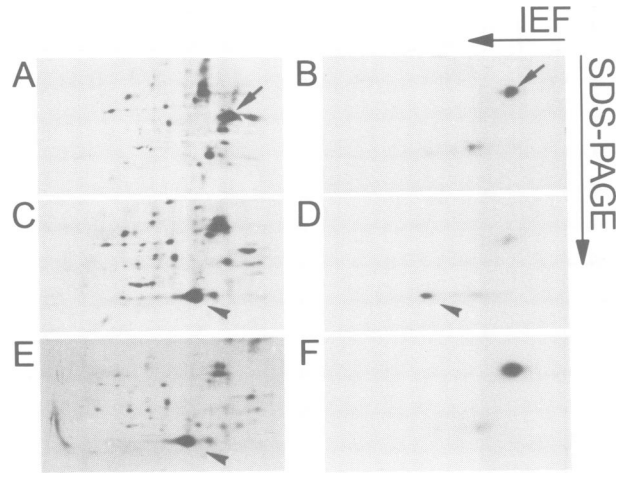

Fig. 4. Two-dimensional gel electrophoresis of cell supernatants obtained after pulse-labeling with [^{35}S]methionine. Cells were stimulated for 90 min with LPS and then transferred to methionine-free medium spiked with [^{35}S]methionine for another 90 min. Thereafter, they were transferred to K^+ buffer + CH + 1 μg/ml alpha-toxin (**A**) or Na^+ buffer + CH + alpha-toxin (**C**) or Na^+ buffer + CH without alpha-toxin for 60 min (**E**). The autoradiographs were developed after 21 days and the prominent 17 kDa entity present in Na^+ buffer supernatants [see (E), arrows] was absent in the K^+ buffer supernatant (A); instead, a prominent 34 kDa polypeptide was present in the K^+ buffer supernatant [(A), arrow] that was absent in the Na^+ buffer supernatants. Recombinant IL-1β and pro-IL-1β were radioiodinated and a mixture of the two markers yielded the autoradiograph (**F**). Appropriate amounts of each marker were then added as a mix to the supernatants of (A) and (C); the autoradiographs were developed after 2 days and are shown in (**B**) and (**D**). Observe the reciprocal intensification of the 17 and 34 kDa spots [arrows, (B) and (D)] resulting from co-electrophoresis of the [^{35}S]methionine-labeled polypeptides and the radioiodinated markers.

radioiodinated IL-1β generated three 17 kDa spots differing slightly in their isoelectric points (Figure 4F). That the three acidic moieties might represent oxidized derivatives of IL-1β was inferred from the finding that the most alkaline spot totally disappeared if radioiodination was performed without protection of -SH groups (not shown). In the next experiment, the marker mix was added to the cell supernatants of gels 4A and 4C. The amounts of radiolabeled markers were adjusted so as to optimize the intensity of the 17 and 34 kDa spots. The autoradiographs were developed after 2 days in order to minimize background, and the relevant parts of the autoradiographs are shown in Figure 4B and D. Co-electrophoresis of the marker mix with the K^+ buffer supernatant led to intensification of the 34 kDa spot, which was thus identified as pro-IL-1β. Co-electrophoresis with the Na^+ buffer supernatant analogously identified the alkaline 17 kDa spot as secreted, mature IL-1β.

In the next experiments, cells were stimulated for 1, 2, 3, 4 or 6 h, and then treated with 1 μg/ml alpha-toxin in the presence of varying K^+ concentrations, and the supernatants were assayed for IL-1β after 40 min. As shown in Figure 5, a clear dose–response relationship was observed in all cases, whereby the efficacy of K^+-dependent inhibition decreased with the duration of LPS stimulation. IL-1β maturation was totally suppressed in cells stimulated for 1 and 2 h by 60 and 90 mM K^+, respectively.

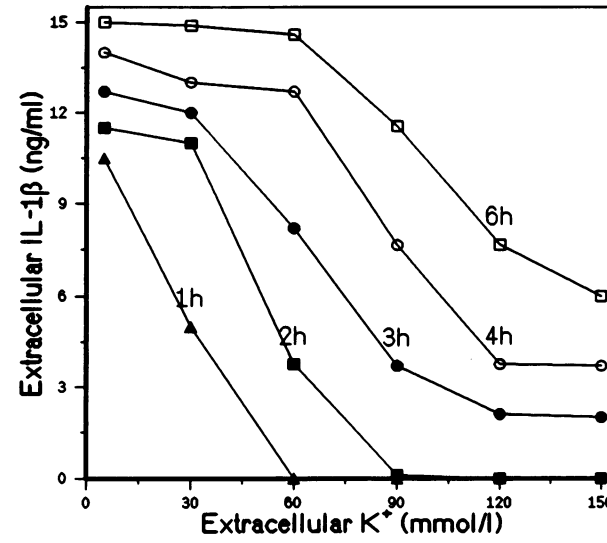

Fig. 5. Inhibition of IL-1β release to cell supernatants by K^+ after LPS stimulation of monocytes for the depicted times and permeabilization with 1 μg/ml alpha-toxin for 45 min.

K^+ depletion of cells by other agents also stimulates IL-1β maturation

To test whether other K^+-depleting agents or manipulations might also trigger IL-1β maturation, monocytes were treated with gramicidin, nigericin, valinomycin or oubain in Na^+ buffer, and IL-1β determined in the supernatants. LPS stimulation was performed for 90 min in order to maximize intracellular accumulation of pro-IL-1β. Thereafter, the respective agents were applied for 60 min together with CH. K^+ determinations indicated ~75% reduction of intracellular K^+ levels by 200 μM ouabain in 90 min (data not shown). Nigericin and valinomycin are known to markedly deplete macrophages of K^+ (Perregaux and Gabel, 1994). In separate experiments, monocytes were depleted of K^+ by brief exposure to hypotonic shock in K^+-free medium. As shown in Figure 6, all treatments resulted in processing and export of IL-1β. In contrast, neither A23187, the Ca-specific ionophore that facilitates influx of Ca^{2+}, nor thapsigargin, an agent that mobilizes Ca^{2+} from intracellular stores, provoked IL-1β secretion. The negative results with A23187 were obtained over the tested range of 0.1–10 μM.

High extracellular K^+ inhibits IL-1β export in LPS-stimulated monocytes

To obtain an indication of whether K^+ movement might also play a role in a physiological setting, monocytes were stimulated with LPS for 90 min and then transferred to K^+ buffer. As shown in Figure 7A, these cells were entirely unable to secrete mature IL-1β. In the depicted experiment, K^+ buffer was removed after 6 h and replaced with Na^+ buffer containing cycloheximide. This led to rapid IL-1β secretion within 30 min. In other experiments, incubation in K^+ buffer was continued for up to 16 h, after which it was similarly found that the cells could process and export their residual content of pro-IL-1β upon transfer to Na^+ buffer. Levels of mature IL-1β were lower in such cases due to gradual leakage of unprocessed pro-IL-1β from the cells in K^+ buffer (not shown).

In the next experiments, monocytes were stimulated for

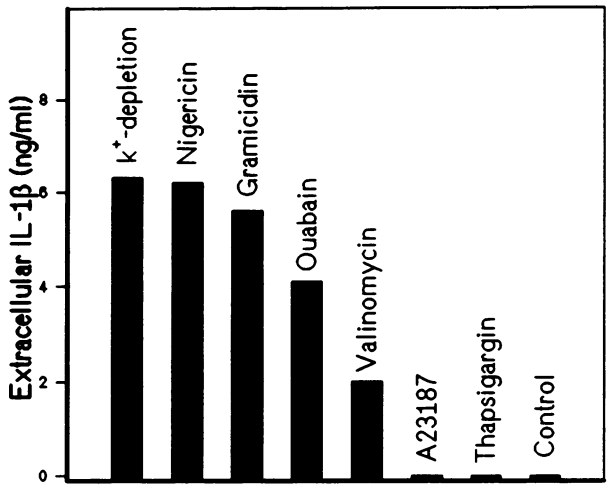

Fig. 6. IL-1β maturation in LPS-stimulated monocytes is triggered by all K^+-depleting maneuvers. Cells were stimulated for 90 min with LPS and then transferred to medium containing 20 μM nigericin, 10 μM gramicidin, 200 μM ouabain, 20 μM valinomycin, 10 μM A231287 or 2.5 μM thapsigargin in the presence of cycloheximide for 90 min. In addition, K^+ depletion was provoked by brief hypotonic shock (first column). Extracellular IL-1β was quantified in cell supernatants. Note the absence of IL-1β maturation provoked by agents that enhance intracellular Ca^{2+} concentrations.

1, 2, 3 or 6 h with LPS, and then transferred to medium containing varying K^+ concentrations for another 18 h. Mature IL-1β was quantified in the supernatants. The results are shown in Figure 7B. As was observed with toxin-permeabilized cells, IL-1β maturation could be inhibited in all cases, but increasing K^+ concentrations were required, depending on the duration of the LPS stimulation phase. After a 6 h stimulation with LPS, IL-1β maturation was 75% inhibited by 150 mM KCl, but little inhibition was observed at 120 mM K^+.

In the next experiment, pro-IL-1β was measured in supernatants. Cells were stimulated with LPS for 90 min and first transferred to Na^+ buffer for 6 h. During this time, export of mature IL-1β occurred, with little liberation of pro-IL-1β. Upon transfer to K^+ buffer, IL-1β maturation was inhibited (see above) and pro-IL-1β was released instead into the supernatants (Figure 7C). These results again showed that arrest of IL-1β maturation was accompanied by export of the precursor to the extracellular medium.

K^+ channel blockers inhibit IL-1β export in LPS-stimulated monocytes

Monocytes were stimulated with LPS for 2 h and two K^+ channel blockers, tetraethylammonium (TEA) and 4-aminopyridine (4-AP), were added. When applied at high concentrations, each blocker alone slightly decreased IL-1β secretion. When applied in combination, however, an almost complete inhibition of IL-1β export was observed (Figure 8).

Discussion

The model systems employed in this study indicate that K^+ ions directly or indirectly influence the IL-1β maturation process. Quantitation of pro-IL-1β and mature IL-1β was possible because the immunoassays employed each exhibited exclusive specificity for the homologous antigen. That ELISA kits measuring mature IL-1β usually do not detect pro-IL-1β was already known (Dinarello, 1992; Herzyk *et al.*, 1992; Bailly *et al.*, 1994), and our results show the opposite to also hold. This fortunate coincidence thus now renders simultaneous quantitation of pro-IL-1β and mature IL-1β possible with the use of commercial ELISA kits. The validity of the assays was confirmed in this study by two-dimensional gel electrophoresis. The results were concordant with immunoassays in showing that monocytes permeabilized with alpha-toxin in the presence of K^+ could no longer secrete mature IL-1β but, instead, leaked pro-IL-1β. The latter finding conformed to what might have been expected from the literature. Thus, it is known that blockade of ICE activity by specific protease inhibitors results in export of pro-IL-1β from LPS-stimulated cells (Thornberry *et al.*, 1992; Jessop *et al.*, 1993). In effect, the action of high K^+ is akin to that of a reversible proteinase inhibitor. An important conclusion is that secretion of the cytokine is not K^+ dependent and not inevitably linked to the processing step.

Membrane permeabilization by alpha-toxin could be done in a controlled fashion (Bhakdi *et al.*, 1993). K^+ does not affect the formation of hexamers (Jonas *et al.*, 1994; and unpublished data). Since the toxin pores did not permit Ca^{2+} flux, the cells were saved from the deleterious consequences of flooding with this potentially toxic cation. On the other hand, cellular Ca^{2+} homeostasis and nucleotide content in the cells were intact enough to guarantee functionality of the IL-1β processing and export machinery. A conceptually similar approach was recently taken by Perregaux and Gabel, who used ATP as the permeabilizing agent. These authors also demonstrated that high K^+ inhibited pro-IL-1β processing (Perregaux and Gabel, 1994). Our data extend those findings to show that ICE inhibition in permeabilized cells occurs *in situ* at varying K^+ concentrations, depending on the duration of the LPS stimulation phase, and that this is accompanied by export of uncleaved pro-IL-1β. Further, our results negate a role for influx of either Na^+ or Ca^{2+} in the regulation of ICE activity.

A basic question then arose regarding the possible role of K^+ levels in intact, LPS-stimulated cells. This question has not been addressed in any investigation hithertofore. Two types of experiments were performed. First, monocytes were stimulated with LPS for 90 min to trigger synthesis of pro-IL-1β without the release of mature IL-1β (Rubartelli *et al.*, 1990). Subsequent transfer of these cells to high K^+ medium was shown to totally block IL-1β maturation. The processing step could, however, rapidly be initiated by transferring the cells back to low K^+ medium. The fact that IL-1β maturation occurred even after 16 h incubation in KCl demonstrated that the export machinery tolerated exposure to high extracellular K^+ for remarkably long periods and provided evidence that K^+ levels indeed influenced IL-1β maturation even in intact cells. At the same time, the experimental protocol presents a long-sought method for quantitatively dissociating pro-IL-1β synthesis from its processing. Other investigators have expended considerable efforts to attain this goal. One method employed stimulation of monocytes with very low doses (50 pg/ml) of LPS (Chin and Kostura, 1993). Overnight culture of monocytes in the presence of

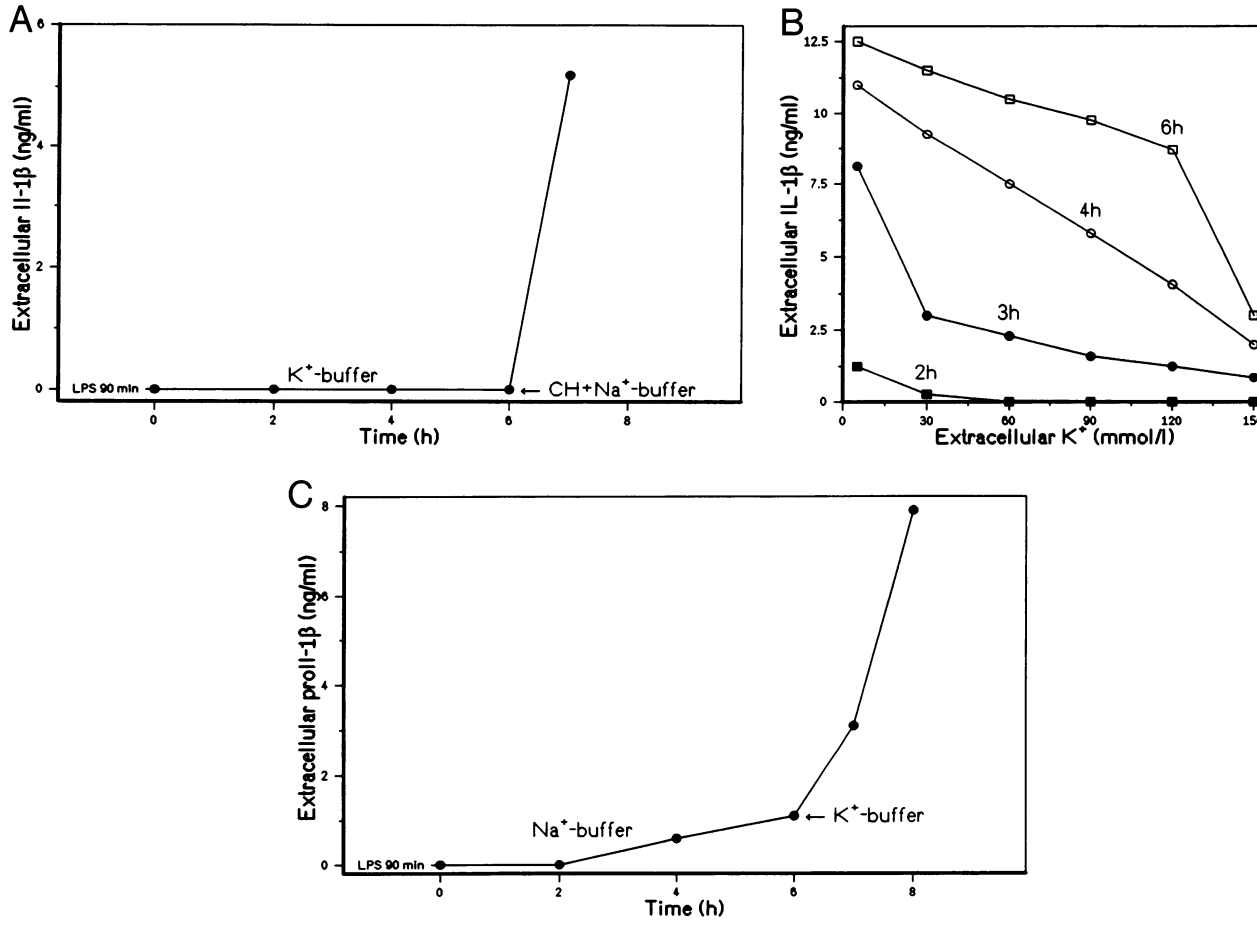

Fig. 7. Inhibition of IL-1β maturation in intact monocytes stimulated with LPS. (**A**) Cells were stimulated with LPS for 90 min and then transferred to K^+ buffer: no IL-1β secretion occurred for 6 h. Thereafter, cells were placed in Na^+ buffer with CH: IL-1β maturation immediately occurred. (**B**) Cells were LPS stimulated for 2–6 h and then transferred to medium containing increasing K^+ concentrations + CH. IL-1β was quantified in supernatants after 16 h. Note the K^+-dependent inhibition of IL-1β secretion in all cases, but increasing K^+ was required as the stimulation phase was prolonged. (**C**) Release of pro-IL-1β from monocytes that were stimulated for 90 min with LPS, then transferred to Na^+ buffer to permit IL-1β maturation, followed by transfer to K^+ buffer to inhibit maturation. Note the leakage or secretion of pro-IL-1β occurring with blockade of maturation.

fetal calf serum probably usually elicits the same response (Couturier *et al.*, 1990). These methods require strict adherence to experimental protocols and are sensitive to disturbances, whereas the procedure described herein is easy and infallible.

The second set of experiments addressed the concentration dependency of K^+-dependent inhibition. Cells were stimulated with LPS for increasing times and then transferred to medium containing varying K^+ concentrations. Measurements of IL-1β in these supernatants after 16 h incubation revealed that the extracellular K^+ levels required to inhibit IL-1β maturation increased with the duration of the LPS stimulation phase. Even after a 6 h stimulation, ongoing IL-1β export could, however, be largely arrested in 150 mM KCl buffer. We propose that K^+ ions directly or indirectly interfere with the interaction of ICE with its substrate, and the concentration of K^+ required for inhibition depends on the intracellular concentrations of enzyme and substrate. It is known that LPS stimulation simultaneously triggers synthesis of pro-IL-1β and ICE, so that the concentrations of these entities probably increase over time (Black *et al.*, 1988). In a physiological setting, this would imply that cells loaded with high levels of pro-IL-1β will be induced to export

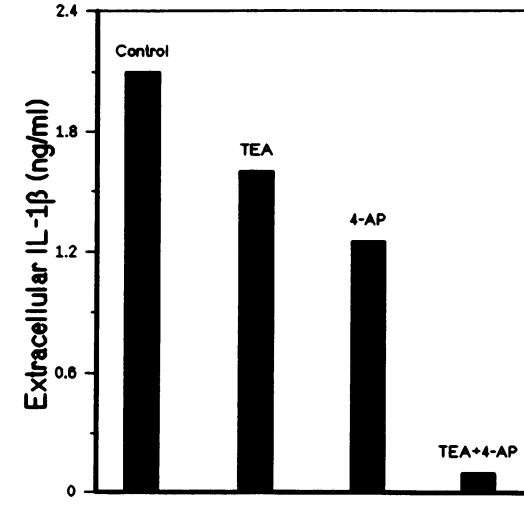

Fig. 8. Inhibition of IL-1β maturation by a combination of the K+ channel blockers TEA and 4-AP. Cells were stimulated with LPS for 2 h. Thereafter, TEA (50 mM) and 4-AP (5 mM) were applied in Na^+ buffer + CH. Extracellular IL-1β was measured after 60 min.

mature IL-1β upon only slight reductions in K^+ concentrations. On the other hand, cells harboring relatively low concentrations of pro-IL-1β will presumably process the cytokine only when they are severely depleted of K^+, such as occurs with the pore-forming agents or K^+ ionophores.

The possibility that K^+ movement is important for IL-1β maturation receives direct and indirect support from other findings. First, B cells stimulated with LPS rapidly synthesize and increase up to 1000-fold the number of voltage-dependent K^+ channels in the plasma membrane (Amigorena *et al.*, 1990; Partiseti *et al.*, 1993). Whether monocytes respond similarly to LPS stimulation is not documented, but we believe it to be likely. The role and biological significance of such dramatic increases in K^+ channels are unknown, but what would be more likely than that they should enhance spontaneous K^+ flux? It is interesting that monocytes contain no Ca^{2+}-activated K^+ channels (Gallin and McKinney, 1988), and this satisfactorily explains why A23187 and thapsigargin do not stimulate ICE activity. The second argument relates to our finding that the K^+ channel blockers TEA and 4-AP also inhibit IL-1β maturation. It is realized that the concentrations required are high, and that only a combination of both agents was really effective. However, in view of the probable necessity to quantitatively block K^+ channels, it is our bias that the results are compatible with K^+ flux being significant. A third and independent finding is that other agents or manipulations causing cellular K^+ depletion also triggered IL-1β maturation. This was already known for nigericin (Perregaux *et al.*, 1992) and has now been extended to valinomycin, ouabain, gramicidin, and cells briefly subjected to hypotonic shock, which also leads to K^+ depletion (Larkin *et al.*, 1983). These findings are in line with the hypothesis that K^+ depletion is a major event leading to IL-1β maturation. Finally, it is most interesting to note that the *in vitro* activity of ICE was reported to be sensitive to high K^+ by the discoverers of this enzyme (Kostura *et al.*, 1989). Also possibly related is the finding that dying cells release IL-1β (Hogquist *et al.*, 1991; Jessop and Hoffman, 1993). Since these cells lose control of their K^+ homeostasis, it is understandable that triggering of IL-1β maturation should occur similarly to the situation with alpha-toxin.

If our proposal that K^+ levels influence cleavage of pro-IL-1β by ICE is correct, this would be the first example for control of a proteolytic process by the major intracellular ion. It is not difficult to envisage how alterations of local K^+ concentrations could occur in intact cells. Increasing the number of K^+ channels in both plasma and intracellular membranes may be generally important. Emerging evidence suggests that compartments with gated K^+ channels exist within cells (Fleischer *et al.*, 1994). Further, fusion between Na^+-rich endosomes and intracellular vesicles may represent a simple mechanism for lowering K^+ concentrations in intracellular compartments. In this context, intracellular pro-IL-1β (Giri *et al.*, 1985; Bayne *et al.*, 1986; Bakouche *et al.*, 1987; Singer *et al.*, 1988) appears to be present in trypsin-resistant vesicles (Rubartelli *et al.*, 1990). K^+ may itself influence the movement of vesicles, akin to its inhibitory effect on endocytosis (Larkin *et al.*, 1983; McVey Ward *et al.*, 1990; Ilondo *et al.*, 1991; Hansen *et al.*, 1993).

It is intriguing to extrapolate the concept of K^+-regulated proteolytic processes to other events. For example, the *ced3* gene product may also be a K^+-regulated protease. Should a homologous protease be present in nucleated cells, this might explain our previous finding that alpha-toxin also triggers apoptosis in activated human lymphocytes, and that DNA degradation in this particular case is inhibitable by high K^+ in the medium (Jonas *et al.*, 1994). It would also be consistent with the report that low K^+ induces apoptosis in cerebellar granule neurons (D'Mello *et al.*, 1993, 1994) and that neither Ca^{2+} influx nor Ca^{2+} mobilization induces apoptosis (Lennon *et al.*, 1992; Duke *et al.*, 1994). Future exploration of the role of the major intracellular ion in controlling vitally important cellular functions could lead to basic recognitions in cell biology.

Materials and methods

Reagents

Chemical reagents were purchased from Sigma (Taufkirchen, Germany). Human recombinant IL-1β (catalog no. C-6112) was obtained from Promocell (Heidelberg, Germany). Human recombinant IL-1β precursor (catalog no. E01-18-1013) was obtained from Cistron Biotechnology (Pine Brook, NJ). ^{125}I was obtained from DuPont de Nemours (Dreieich, Germany). *Staphylococcus aureus* alpha-toxin was purified as described previously (Bhakdi *et al.*, 1989).

K^+ buffer contained 150 mM KCl, 5 mM NaH_2PO_4, 10 mM HEPES, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 1% bovine serum albumin (BSA). The pH was adjusted to 7.4 with KOH. Na^+ buffer contained 150 mM Na^+, 5 mM KH_2PO_4, 10 mM HEPES, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1% BSA. The pH was adjusted to 7.4 with NaOH. Buffers containing various concentrations of K^+ were prepared by appropriately combining the above two buffers.

Production of radioiodinated pro-IL-1β and IL-1β

Recombinant IL-1β or pro-IL-1β was transferred to phosphate-buffered saline (PBS) (125 mM Na^+, 25 mM NaH_2PO_4, pH 7.4). Sulfhydryl groups were blocked with *N*-ethylmaleimide (15 min, room temperature). For radioiodination, 0.5 μg of protein and 200 μCi ^{125}I (as NaI) were incubated in siliconized glass tubes coated with 5 mg 1,3,4,6-tetrachloro3,6-diphenylgycoluril ('Jodogen'; Sigma) for 5 min at room temperature. The reaction was stopped by transferring the mixture into fresh vials containing 10 mM sodium metabisulfite. BSA was added to 0.1% and the labeled protein purified by gel filtration (PD10 columns; Pharmacia, Freiburg, Germany).

Preparation and activation of human monocytes

Human peripheral blood mononuclear cells were obtained from fresh blood donated by healthy volunteers. Monocytes were isolated as described previously (Denholm and Wolber, 1991). The monocytes were resuspended at 1×10^6 cells in RPMI 1640 (Gibco) supplemented with 10% antologous human serum, 1 mM L-glutamine and 100 U/ml penicillin–streptomycin and plated in 24-well plates (Nunc). After incubation for 1 h at 37°C, non-adherent cells were removed and the remaining cells were activated with 1 μg/ml LPS (*Escherichia coli* serotype 026:BH6; Sigma).

Quantification of pro-IL-1β and IL-1β

Quantification of pro-IL-1β and IL-1β was undertaken following the instructions supplied by the manufacturers of the ELISA kits. The IL-1β ELISA was supplied by Medgenix (catalog no. 40 12100; Medgenix Diagnostic GmbH, Ratingen, Germany) and the pro-IL-1β ELISA by Cistron (catalog no. 03-1000; Cistron Biotechnology, Pine Brook, NJ). The cytokines were quantified in cell culture supernatants or in detergent lysates of the monocytes. The cell supernatants were first recovered for the determination of released cytokines. Adherent cells were then lysed with 0.1 ml of a 0.2% aqueous Triton X-100 solution. Control experiments showed that the presence of detergent at this concentration did not affect the performance of the ELISAs. The respective calibration curves were prepared with or without detergent by using the standards supplied with

the kits. Each determination was performed in duplicate and the averages were taken.

K^+ depletion of cells by hypotonic shock was performed as described previously (Larkin *et al.*, 1983). K^+ and Ca^{2+} flux measurements were performed by flame photometry (Walev *et al.*, 1993).

Two-dimensional gel electrophoresis

Monocytes were stimulated with 1 μg/ml LPS for 90 min and then incubated in methionine-free RPMI medium containing 1 mCi [^{35}S]-methionine for another 90 min. The cells were then washed and re-incubated with buffer containing 1 μg/ml alpha-toxin for 45 min at 37°C. The supernatants were recovered and subjected to isoelectric focusing, followed by SDS–PAGE, according to conventional procedures (Reske and Weitzel, 1985).

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